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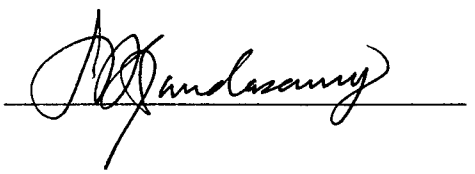

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13. ABSTRACT *(Maximum 200 Words)*

The peptidyl-prolyl isomerase Pin1 has been implicated in regulating cell cycle progression. Pin1 was found to be required for the DNA replication checkpoint in *Xenopus laevis*. Egg extracts depleted of Pin1 inappropriately transited from G_2 to M phase of the cell cycle in the presence of the DNA replication inhibitor aphidicolin. This defect in replication checkpoint function was reversed after addition of recombinant wild type Pin1, but not an isomerase-inactive mutant, to the depleted extract. Premature mitotic entry in the absence of Pin1 was accompanied by hyperphosphorylation of cdc25, activation of cdc2/cyclin B, and generation of MPM-2 epitopes. Therefore, Pin1 appears to be required for the checkpoint delaying the onset of mitosis in response to incomplete replication.

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Katharine Uphilder 25 July 2000

PI - Signature Date

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Introduction

In the progression to transformation, many cancer cells disable the surveillance mechanisms that sense damaged DNA and arrest the cell cycle to repair the damage before initiating mitosis. Such checkpoints normally operate by prolonging the cytoplasmic sequestration of cdc25C and thereby preventing its activation of the mitotic kinase cdc2/cyclin B. We are studying the mechanism of action of a novel cell cycle regulator, Pin1, which influences the progression from G2 to M phase. At the time the project was proposed, the affinity of each of Pin1 domains for phospho S/T-P sequences had not yet been recognized. We had made the observation that several mitotically phosphorylated proteins from *Xenopus* extracts bound Pin1. Among these, cdc25 was of particular interest because of its role as a mitotic initiator in activating the mitosis-promoting factor (MPF), cdc2/cyclin B, and the correlation of its upregulation in breast cancers with worsened prognoses. Furthermore, the mitotic cell cycle defect that occurs in extracts depleted of Pin1 binding proteins is complemented by the addition of recombinant phosphorylated cdc25. These observations were the premise for my proposed project of investigating cdc25's oncogenic role in breast cancer using Pin1 as a novel molecular probe.

Body

Initially the investigation of cdc25's oncogenic role using Pin1 required elucidation of biological nature of Pin1's own function to allow interpretation and synthesis of any prior and subsequent observations pertaining to cdc25. This was the goal of the first technical objective, and the results are summarized below.

The novel cell cycle regulator, Pin1, appears to have a function in the regulation of the G_2 to M-phase transition. This peptidyl-prolyl isomerase was originally identified in yeast-two hybrid screens as a protein that binds the fungal mitotic kinase, NIMA. More recently, it has been recognized that Pin1 also associates with cdc25C and several other mitotic phosphoproteins. Although it remains to be demonstrated that these interactions are biologically relevant, Pin1 protein clearly influences cell cycle dynamics. Over-expression of the protein is deleterious in the budding yeast, *Saccharomyces cerevisiae* and causes a G_2 arrest in HeLa cells and in *Xenopus laevis* egg extracts, implicating the protein as a negative regulator of mitotic initiation. The budding yeast Pin1 homologue, ESS1, is encoded by an essential gene; *ess1* deletion mutants exhibit terminal mitotic arrest suggestive of a requirement for Pin1 in mitotic exit. ESS1 has been connected genetically and biochemically to transcriptional regulation and RNA processing in yeast, although it is not clear the degree to which these functions contribute to the mitotic failure in the deletion strain.

The *Xenopus* Pin1 homologue was isolated by low stringency hybridization screening of a *Xenopus* gastrula cDNA library using a human Pin1 probe. The inserts of three independently isolated clones each encoded an identical open reading frame (*xPin1*). The predicted polypeptide sequence shared 89% identity with human Pin1 and greater than 45% identity with each of the eukaryotic parvulins over its full length of 159 residues. Recombinant xPin1 was purified from bacteria and used to generate polyclonal antiserum that recognized a single protein of 18kDa in *Xenopus* egg extracts. The concentration of Pin1 in egg extracts was estimated to be 20 ng/μL, and this did not change throughout the cell cycle.

We examined Pin1 function in isolated cell cycle transitions in *Xenopus* egg extracts, a transcriptionally inactive cell-free system that avoids the potential complications introduced by Pin1's effects on transcriptional events that might affect cell cycle progression. The terminal arrest in mitosis of yeast cells lacking Pin1 suggested a function for the protein in mitotic exit, but immuno-depletion of the protein from *Xenopus* extracts revealed no defect either in the M-G_1 transition or in DNA replication. Alternatively, the yeast mitotic arrest could be triggered in response to genetic instability or chromosomal defects incurred earlier in the cell cycle as a consequence of Pin1 depletion. In support of this interpretation, interphase-arrested extracts entered mitosis more rapidly when Pin1 was removed. Inclusion of high concentrations of DNA extended the duration of interphase in mock-depleted but not Pin1-depleted extracts, indicating a failure of a negative regulatory influence at the G_2-M transition in extracts lacking Pin1. Pin1-depleted extracts not only entered mitosis inappropriately, but they also failed to exit M-phase. This might be expected if the replication checkpoint were disabled in Pin1-depleted extracts; premature mitosis in the presence of incompletely replicated DNA would activate M-phase failsafe mechanisms to prevent segregation of damaged chromosomes. Indeed, Pin1-depleted extracts were refractory to treatment with the DNA polymerase inhibitor, aphidicolin. Mock-depleted extracts effectively postponed mitotic entry in response to replication inhibition, while depletion of Pin1 from extracts or addition of caffeine (a treatment that disables the replication checkpoint) prevented this aphidicolin-induced cell cycle delay. Supplementation of Pin1-depleted extracts with recombinant xPin1 restored the caffeine-sensitive replication checkpoint.

Pin1's effects on cell cycle kinetics might be mediated through its established association with mitotic phosphoproteins. For this reason, we examined the status of several Pin1-binding proteins in extracts depleted of Pin1. In all of our assays, the premature mitosis observed in Pin1-

depleted extract was indistinguishable from that occurring in the presence of caffeine. Hyperphosphorylation of cdc25, increased H1 kinase activity, and appearance of MPM-2 epitopes accompanied microscopically observed mitotic entry in both cases. Therefore, Pin1 is not required for MPM-2 epitope generation or for the ability of these phosphoproteins to regulate mitotic progression. Instead, precocious activation of cdc25 may be the direct consequence of Pin1 removal.

Recently it was reported that Pin1 antagonizes in vitro phosphorylation of the mitotic regulators, cdc25, myt1 and wee1 by cdc2/cyclin B. Although the ability of Pin1 to bind mitotic phosphoproteins appears to be important for this inhibition, association alone is not sufficient for endogenous Pin1 function. We introduced a point mutation (C109A) into *Xenopus* Pin1 which compromised the prolyl isomerase activity of the enzyme more than ninety percent without diminishing its protein binding avidity. This mutant was incapable of restoring the checkpoint response in Pin1-depleted extracts when added to achieve concentrations sufficient for complementation by wild type xPin1, despite its robust binding to NIMA and hyperphosphorylated cdc25 in pull-down assays. Furthermore, *Xenopus* Pin1 complements the lethality of *ess1* mutants in budding yeast, but the C109A mutant is inactive in the complementation assay. Together these observations indicate that Pin1 is functionally conserved and that its catalytic activity is required for its checkpoint role in *Xenopus* and its essential function in *S. cerevisiae*.

Collectively, these results offer compelling evidence that Pin1 is required for the replication checkpoint signal in *Xenopus* and establish the protein as a temporal regulator of cell cycle progression. Target-specific inhibition of mitosis-promoting kinase activity provides a biochemical mechanism for Pin1's role in enabling the replication checkpoint. Pin1-mediated inhibition of cdc25 hyperphosphorylation likely acts in concert with 14-3-3-mediated cytoplasmic sequestration of the phosphatase to prolong G_2 by preventing functional interaction of cdc25 with cdc2 under checkpoint conditions. This function is consistent with previous observations that Pin1 over-expression delays mitotic initiation and that *S. cerevisiae ess1* deletion mutants arrest in mitosis, an expected consequence of premature mitotic entry. In the absence of DNA perturbation, consequences of Pin1-removal may not be manifested; this would explain the lack of apparent phenotype in Pin1-null *Drosophila* mutants. Our demonstration of the essential role of Pin1 in the replication checkpoint establishes a position for endogenous Pin1 in the eukaryotic cell cycle regulatory network.

Although Pin1 remains circumstantially linked with cdc25 in regulating the G2-M transition, its usefulness as a molecular probe of cdc25's oncogenic mechanism is limited by the lack of mechanistic understanding of Pin1's checkpoint function. Individual phosphorylation site mutations in cdc25 were generated and assessed in their abilities to bind Pin1 before or after incubation with mitotic extract. There was not a single target site which, when removed, resulted in a loss of interaction. In light of the recent recognition that Pin1 binds a number of phospho-peptides, we expect that the cdc25 binding initially observed was dramatic precisely because cdc25 is such a heavily phosphorylated protein in the mitotic extract. Because we were unable to show any endogenous association of Pin1 with cdc25, despite many attempts of co-immunoprecipitation, our efforts in this category have been re-directed toward the characterization of a potential mammalian homologue of NIMA. At the present time this kinase, which was identified in a database search, has met several criteria that one might expect to be fulfilled by a mammalian NIMA. Importantly, it does bind Pin1 in vitro as well as cause abnormal chromatin condensation from any cell cycle stage upon transfection into mammalian cells. Ongoing generation of stable cell lines expressing inducible vectors encoding both sense and antisense clones, as well as generation of antisera in rabbits, should provide systems and reagents for a more complete assessment of the kinase's function. Furthermore, domain-mapping

studies are underway to determine whether the chromatin-condensation causing activity is separable from Pin1-binding ability.

We expect that this research will provide an important piece in assembling the puzzle of G_2 regulation and transduction of signals from chromatin to mitotic initiators. We have demonstrated a positive correlation between mammalian Pin1 expression and the proliferative status of cells and tissues. Furthermore, Pin1 was dramatically over-expressed in each of six human lung tumors relative to adjacent normal tissue. A number of the solid tumors in which Pin1 levels are elevated also exhibit p53 mutations. The cell cycle is actually extended in such tumor cells, perhaps as a consequence of Pin1 upregulation as the cells struggle to delay entry into mitosis until DNA is replicated and repaired. In this situation, inhibition of Pin1 would inactivate the delay mechanism and prompt cells to proceed into catastrophic mitosis. Pin1 is an especially attractive target for anti-cancer therapeutics in light of our observation that the prolyl isomerase activity of the protein appears to be important for its checkpoint function. Because protein binding is not sufficient for Pin1 function, disabling the catalytic activity of the isomerase with small molecules ought to achieve the desired effect without necessitating the disruption of a protein-protein interaction. Inhibition of Pin1's prolyl isomerase activity might selectively sensitize tumor cells bearing intrinsic defects in components of the checkpoint machinery to destruction by conventional anticancer agents that interfere with DNA replication or induce DNA damage.

Key Research Accomplishments

-Cloning of *Xenopus* Pin1
-Purification of recombinant xPin1 protein
-Generation of polyclonal anti-xPin1 antibody
-Demonstration that Pin1 is not required for mitotic exit or DNA replication
-Demonstration that Pin1 does regulate the timing of mitotic entry
-Demonstration that Pin1 is required for the replication checkpoint
-Generation of Pin1 C109A mutant
-Analysis of C109A's ability to bind NIMA and cdc25
-Determination that C109A is incapable of restoring Pin1's checkpoint function
-Generation of cdc25 phosphorylation site mutants
-Analysis of their abilities to bind Pin1

Reportable Outcomes

Publications

Katharine E. Winkler, Katherine I. Swenson, Sally Kornbluth, Anthony R. Means. 2000. Requirement of the Prolyl Isomerase Pin1 for the Replication Checkpoint. *Science,* 287:1644-1647.

Jing Yang, Katharine Winkler, Minoru Yoshida, and Sally Kornbluth. 1999. Maintenance of G_2 arrest in the *Xenopus* oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import. *EMBO,* 18: 2174-2183.

Abstracts

The Replication Checkpoint Requires Pin1. *Keystone Symposium,* January 2000: "Cancer, Cell Cycle and Therapeutics" (with Katherine I. Swenson, Sally Kornbluth and Anthony R. Means).

Pin1, A Novel Cell Cycle Regulator which Functions in the Transition from G_2 to M Phase. *Gordon Research Conference,* July 1999: "Molecular and Genetic Basis of Cell Proliferation" (with Sally Kornbluth and Anthony R. Means).

Invited Lectures

*Pin*ning Down the Cell Cycle. Harold M. Weintraub Graduate Student Award Symposium. May 2000.

The Replication Checkpoint Requires the Prolyl Isomerase, Pin1. *Duke University Biological Sciences Graduate Student Symposium.* October 1999.

The Replication Checkpoint Requires the Prolyl Isomerase, Pin1. *Duke University Department of Pharmacology and Cancer Biology Departmental Retreat.* April 1999.

Honors

Recipient, first annual Harold M. Weintraub Graduate Student Award. 2000.

The Replication Checkpoint Requires Pin1

Katharine E. Winkler*, Katherine I. Swenson, Sally Kornbluth, and Anthony R. Means, Department of Pharmacology and Cancer Biology, Duke University, Durham, NC, USA, 27710

Over-expression of the peptidyl-prolyl isomerase, Pin1, causes a G_2 arrest in various systems implicating it as a negative regulator of mitotic initiation. Furthermore, a requirement for Pin1 in mitotic exit is suggested by the terminal mitotic arrest observed in *Saccharomyces cerevisiae* null for the Pin1 homologue. We define a role for Pin1 in the vertebrate cell cycle by examining its function in through immunodepletion of the protein from *Xenopus laevis* egg extracts. This model system provides the opportunity to evaluate the contribution of Pin1 in isolated cell cycle transitions.

Pin1-depleted and mock-depleted extracts are equally capable of exiting M phase, and Pin1-depletion has no effect on DNA replication. Thus, Pin1 is not required for the progression from M phase into interphase in this system. However, Pin1-depleted extracts are consistently accelerated their passage through the G_2/M transition in comparison with mock-depleted extracts. This difference in timing of mitotic entry is exaggerated in the presence of unreplicated DNA, which triggers the replication checkpoint to postpone M phase in mock-depleted extracts. Pin1-depleted extracts fail to halt mitotic entry in response to the DNA polymerase inhibitor, aphidicolin. The addition of recombinant Pin1 restores the appropriate G_2 delay response in Pin1-depleted extracts. These experiments demonstrate that Pin1 is an essential component of the replication checkpoint signal in *Xenopus*, thereby establishing a novel biological function for the endogenous protein.

Katharine Winkler
919 681 6236
Keystone Conference
A2: Cancer, Cell Cycle and Therapeutics
Session3

Gordon Research Conference Abstract
Molecular and Genetic Basis of Cell Proliferation
July 1999

Pin1, a peptidyl prolyl isomerase, participates in the regulation of the G2/M transition in *Xenopus* egg extracts.

Katharine E. Winkler, Sally Kornbluth, Anthony R. Means
Department of Pharmacology and Cancer Biology
Duke University

Cancerous growth is fundamentally dependent on the escape from normal cell cycle control. A group of highly conserved proteins cooperate to regulate cell cycle transitions by ensuring high fidelity replication and subsequent compartmentalization of the genome. We are studying the mechanism of action of a novel cell cycle regulator which influences the progression from G2 to M phase. This regulator, Pin1, consists of an N terminal WW domain and a C terminal peptidyl prolyl isomerase domain. Pin1 is found in all eukaryotes, and in budding yeast the homolog is essential where its inactivation appears to cause a mitotic arrest. Although it has been proposed that the WW protein interaction domain binds a motif that includes phosphorylated serine or threonine followed by proline, and that the isomerase activity is also specific for prolines in this context, the biological function of Pin1 in vertebrates remains obscure.

The current study demonstrates that Pin1 functions in the control of the G2/M transition in *Xenopus laevis* egg extracts. We show that extracts depleted of Pin1 protein enter mitosis prematurely, a phenotype which is dramatically potentiated under circumstances in which the replication checkpoint has been triggered. Our preliminary data show that, in the presence of unreplicated DNA, mock depleted extracts delay entry into mitosis by more than an hour relative to Pin1 depleted extracts which proceed through this transition with similar timing to mock-depleted extracts treated with caffeine to override the checkpoint. While diminution of the endogenous Pin1 protein appears to abrogate the replication checkpoint in our system, addition of excess Pin1 to extracts significantly prolongs interphase by delaying the entry into mitosis, which is suggestive of an inappropriately initiated G2 checkpoint. The complementary effects of addition/removal of Pin1 in the control of the G2 transition strongly implicate Pin1 as a cell cycle regulator . We are unable to demonstrate any requirement for Pin1 in the transition out of M phase; rather than contradict the yeast deletion phenotype of mitotic arrest, we offer the alternate interpretation that the arrest seen in yeast is the result of an intact mitotic checkpoint which has been triggered due to a premature mitosis (prior to completion of replication) resulting from Pin1 inactivation.

[K. Kusano, R. Miledi, J. Stinnakre, *J. Physiol (London)* **328**, 143 (1982)] and 101 mM (based on the fitting of the Nernst plot in Fig. 2E), respectively.
13. However, the possibility exists that Kcv functions as a subunit, which up-regulates the activity of endogenous K^+ channels in oocytes. To exclude this possibility, we constructed a site-specific mutation in the selectivity filter sequence of Kcv by replacing Phe^{66} (F66) with Ala (A). If Kcv is a channel protein, the mutant protein should, by analogy to the Shaker channel, form a channel unable to conduct K^+ currents (*1*). Using standard voltage-clamp assays, nine oocytes expressing KcvF66A had currents similar in kinetics to those of H_2O-injected oocytes with no additional K^+ conductance compared to the H_2O-injected control cells. Hence, the absence of a prominent K^+-selective current in KcvF66A-expressing oocytes confirms that Kcv functions as a channel protein in oocytes and that the observed currents are not due to activation of endogenous channel proteins.
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16. Concentration for half-maximal inhibition estimated by fitting Michaelis-Menten type kinetics to data.
17. The adamantyl group of amantadine is believed to interact with the hydrophobic lining of the M2 pore, whereas the ammonium group forms H bonds with the imidazole ring of His^{37} [C. S. Gandhi *et al.*, *J. Biol. Chem.* **274**, 5474 (1999)].
18. Voltage-dependency of inhibition by Ba^{2+} was analyzed quantitatively based on a Woodhull block model [A. M. Woodhull, *J. Gen. Physiol.* **61**, 687 (1973)] by fitting data of relative block to

$$\left(1 - \frac{I}{I_o}\right) = \frac{b_{max}}{1 + \frac{k^0}{B_a} e^{(z\delta F/RT)}}$$

where I_o is control and I blocked current, b_{max} the maximal block, B_a the concentration of Ba^{2+}, k^0 the dissociation constant of the blocking reaction at voltage = 0 mV, δ the fraction of the electrical field crossed by Ba^{2+}, and $z = 2$ the valence of the blocking ion. R, T, and F have their usual thermodynamic meaning. Fitting yields for 1 mM Ba^{2+}: $b_{max} = 0.94$, $\delta = 0.9 \pm 0.03$, and $k^0 = 660 \pm 12$ μM ($n = 3$ oocytes).
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20. J. L. Van Etten, D. E. Burbank, Y. Xia, R. H. Meints, *Virology* **126**, 117 (1983).
21. About 1200 plaque-forming units of virus PBCV-1, or a small plaque variant P1210 [D. Landstein, D. E. Burbank, J. W. Nietfeldt, J. L. Van Etten, *Virology* **214**, 413 (1995)], were mixed with 10^8 host cells (*Chlorella* strain NC64A) and warm MBBM top agar containing various concentrations of ion-channel inhibitors. The mixture was layered onto MBBM nutrient agar (*22*). After incubating for 2 days at 25°C, viral plaques were counted. Potassium concentration of MBBM growth medium was 1.3 mM.
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27. We thank H. Terlau, D. Gradmann, and M. Blatt for helpful discussions. Supported in part by MURST in the framework of Cofin-99, NIH grant GM32441 to J.V.E., Small Business Innovative Research grant GM41333 to M.N., Telethon grants (#971 and #296.bi) to D.D. and A.M., and a traveling grant by the SmithKline Beecham foundation to B.P.

3 November 1999; accepted 13 January 2000

REPORTS

Requirement of the Prolyl Isomerase Pin1 for the Replication Checkpoint

Katharine E. Winkler, Katherine I. Swenson, Sally Kornbluth, Anthony R. Means*

The peptidyl-prolyl isomerase Pin1 has been implicated in regulating cell cycle progression. Pin1 was found to be required for the DNA replication checkpoint in *Xenopus laevis*. Egg extracts depleted of Pin1 inappropriately transited from the G_2 to the M phase of the cell cycle in the presence of the DNA replication inhibitor aphidicolin. This defect in replication checkpoint function was reversed after the addition of recombinant wild-type Pin1, but not an isomerase-inactive mutant, to the depleted extract. Premature mitotic entry in the absence of Pin1 was accompanied by hyperphosphorylation of Cdc25, activation of Cdc2/cyclin B, and generation of epitopes recognized by the mitotic phosphoprotein antibody, MPM-2. Therefore, Pin1 appears to be required for the checkpoint delaying the onset of mitosis in response to incomplete replication.

The peptidyl-prolyl isomerase (PPIase) Pin1 affects cell cycle transitions. Originally identified in yeast–two hybrid screens as a protein that binds to and suppresses the toxicity of the fungal mitotic kinase Never In Mitosis A (NIMA), Pin1 is present in all eukaryotic cells examined (*1–4*). Although Pin1 is an abundant protein, the expression of which does not change during the cell cycle (Fig. 1), it clearly influences cell cycle dynamics. Overexpression of Pin1 is deleterious in the budding yeast *Saccharomyces cerevisiae* and causes a G_2 arrest in HeLa cells and in *Xenopus laevis* egg extracts, suggesting that the protein negatively regulates the initiation of mitosis (*1, 3*). The budding yeast Pin1 homolog ESS1 is encoded by an essential gene; *ess1* deletion mutants exhibit terminal mitotic arrest, suggesting a requirement for Pin1 in mitotic exit (*4, 5*). In contrast, Pin1 is not critical for any readily observable function in *Drosophila melanogaster* (*2*) or mouse (*6*).

In vitro, Pin1 binds a subset of mitotic proteins containing a motif composed of a phosphoserine or phosphothreonine residue followed by a proline residue (*3, 7–9*) that is also recognized by the MPM-2 monoclonal antibody (*10, 11*). Among these potential cell cycle targets, only substoichiometric interaction of Pin1 with the mitotic phosphatase Cdc25C has been demonstrated in vivo (*12*); thus, it is unclear whether the numerous phosphoproteins associated with Pin1 in vitro are biologically relevant targets for Pin1 in vivo. Endogenous Pin1 protein has been implicated in transcriptional regulation and RNA processing in yeast (*5, 13–16*) and in mediating the association of phosphorylated tau with microtubules in brain extracts (*17*). The relation of these functions to control of the cell cycle remains unclear, and events regulated by Pin1 that influence the cell cycle have yet to be defined. We examined Pin1 function in *Xenopus* egg extracts that are transcriptionally inactive, thus allowing us to avoid possible effects of Pin1 on transcriptional events that might affect cell cycle progression. This model system provided the opportunity to focus on specific cell cycle transitions and thereby evaluate the contribution of Pin1 protein to each transition.

The *Xenopus* Pin1 homolog was isolated by low-stringency hybridization screening of a *Xenopus* gastrula cDNA library with a human Pin1 probe (*18*). The inserts of three independently isolated clones each encoded an identical open reading frame (*xPin1*). The predicted polypeptide sequence shared 89% identity with human Pin1 and >45% identity with each of the eukaryotic parvulins over its full length of 159 residues. Recombinant xPin1 was purified from bacteria (Fig. 1A) and used to generate polyclonal antiserum that recognized a single protein of 18 kD in *Xenopus* egg extracts (Fig. 1B). The concentration of Pin1 in egg extracts was estimated to be 20 ng/μl, or ~1 μM, and this did not change throughout the cell cycle (Fig. 1C).

The mitotic arrest observed in yeast lacking ESS1 suggested a function for the protein in mitotic exit. To test this directly, we used cytostatic factor–arrested egg extracts (CSF extracts) (*19*) to examine the consequences of the removal of Pin1 on mitotic exit and DNA replication. CSF extracts, generated in the presence of EGTA to prevent calcium-dependent degradation of cyclin B, exhibit high H1 kinase activity and other hallmarks of normal M phase arrest. Calcium addition, which recapitulates a physiological consequence of fertilization, causes the extracts to proceed into interphase, characterized by nuclear envelope formation, chromatin

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decondensation, and initiation of replication. When CSF extracts were immunodepleted of Pin1 (*20*), we found that Pin1-depleted and mock-depleted extracts were equally capable of exiting the M phase (Fig. 2, A and B). Furthermore, Pin1 depletion had no effect on DNA replication (Fig. 2C). Thus, in this cell-free system, Pin1 appears not to be required for either S phase or the M to G_1 transition.

Mitotic arrest observed in *ess1* mutant yeast could be the consequence of a premature mitotic entry triggered in the absence of Pin1. To examine the effects of Pin1 depletion on the isolated G_2 to M transition, we used both interphase extracts that were induced to enter M phase by the addition of exogenous cyclin B protein, and cycling extracts, which intrinsically oscillate between S and M phases of the cell cycle (*21*). Microscopic examination of nuclei, coupled with the measurement of Cdc2-cyclin B1–catalyzed histone H1 phosphorylation, revealed that both types of extract, when depleted of Pin1, entered mitosis more rapidly than did control extracts (Fig. 3, A through D). Although the absolute timing of mitotic entry varied from extract to extract, removal of Pin1 consistently accelerated the transition into M phase.

We explored the possibility that the premature mitosis in Pin1-depleted extract was due to the failure of a negative regulatory influence at the G_2 to M transition. The duration of interphase in *Xenopus* extracts can be prolonged by supplementing extracts with high concentrations of sperm chromatin, which increases the time required for DNA synthesis (*22*). The presence of unreplicated sperm DNA triggers the G_2 replication checkpoint that delays mitotic initiation by preventing activation of Cdc2 (*22–24*). The effects of low and high concentrations of sperm in Pin1-depleted or mock-depleted extracts were compared (Fig. 3D). The higher DNA concentration caused a G_2 delay in mock-depleted extracts. However, this delay was greatly reduced in Pin1-depleted extracts. Thus, the difference in timing of mitotic entry observed between mock-depleted and Pin1-depleted extracts may reflect the inability of Pin1-depleted extracts to halt mitotic entry in the presence of unreplicated DNA. Notably, when the concentration of DNA was low, the transition out of mitosis into interphase occurred normally, even without Pin1. In contrast, the Pin1-depleted extract that was supplemented with DNA to achieve a high concentration of chromatin failed to exit mitosis. It is possible that the M phase arrest in these extracts occurs because mitosis is initiated in the presence of unreplicated DNA, and therefore, M phase fail-safe mechanisms are triggered to prevent segregation of damaged chromosomes.

To test the hypothesis that the operation of the replication checkpoint requires Pin1, we suspended replication with the DNA polymerase inhibitor aphidicolin. In mock-depleted extracts, aphidicolin treatment postponed mitotic entry as expected (Fig. 4A). Depletion of Pin1 from extracts or addition of caffeine [a treatment that disables the replication checkpoint (*22, 25–27*)] prevented the aphidicolin-induced cell cycle delay. Supplementation of Pin1-depleted extracts with recombinant xPin1 restored the G_2 delay elicited by aphidicolin (Fig. 4B), and the delay remained caffeine sensitive in the reconstituted extract (*28*). This indicated that Pin1 itself is an essential component of the replication checkpoint in *Xenopus*.

Pin1's effects on cell cycle kinetics might be mediated through its established association with mitotic phosphoproteins (*3, 12*). For this reason, we examined the status of several Pin1-binding proteins in extracts depleted of Pin1. In all of our assays, the premature mitosis observed in Pin1-depleted extract was indistinguishable from that occurring in the presence of caffeine. Hyperphosphorylation of Cdc25, increased H1 kinase activity, and the appearance of MPM-2 epitopes (Figs. 3A and 4C) accompanied microscopically observed mitotic entry in both cases. Therefore, Pin1 is not required for MPM-2 epitope generation or for the ability of these phosphoproteins to regulate mitotic progression. Instead, precocious activation of Cdc25 may be the direct consequence of Pin1 removal.

Recently, it was reported that Pin1 antagonizes in vitro phosphorylation of the mitotic regulators Cdc25, Myt1, and Wee1 by Cdc2/cyclin B (*29*). Although the ability of Pin1 to bind mitotic phosphoproteins appears to be important for this inhibition, association alone is not sufficient for endogenous Pin1 function. We introduced a point mutation [Cys^{109} → Ala^{109} (C109A)] into *Xenopus*

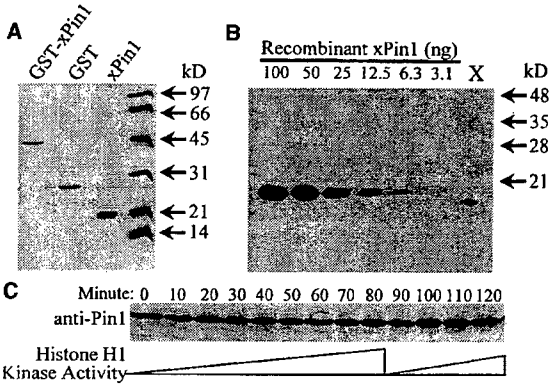

Fig 1. Identification of the *Xenopus laevis* Pin1 homolog. **(A)** Recombinant xPin1 was purified from bacteria as a glutathione *S*-transferase (GST)–xPin1 fusion protein and eluted by cleavage from the GST with thrombin. Coomassie blue staining of 1 μg of each indicated protein separated by SDS–polyacrylamide gel electrophoresis is shown. **(B)** Using antiserum to xPin1 (diluted 5000-fold), we recognized a single protein in *Xenopus* egg extract; 0.5 μl of extract was loaded in lane X. **(C)** The Pin1 content in *Xenopus* extracts (1 μl extract per lane) was visualized by immunoblot over the course of two cell cycles, as assessed by histone H1 kinase activity (*35*) and indicated schematically below the gel lanes.

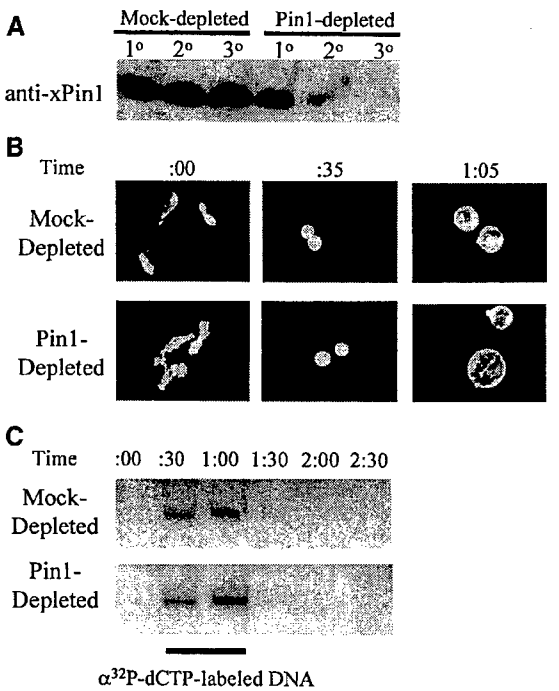

Fig. 2. Exit from mitosis in *Xenopus* extracts lacking Pin1. **(A)** Immunoblot of Pin1 remaining in CSF extracts (*33, 36*) after each stage of immunodepletion. Three successive treatments (1°, 2°, and 3°) removed >95% of Pin1 (*20*). **(B)** 3° depleted extracts were supplemented with demembranated sperm chromatin (*37*) and adenosine 5′-triphosphate (ATP) regenerating mix (*32*) and released from CSF arrest with 400 μM $CaCl_2$. At various times, measured with respect to the time of $CaCl_2$ addition in hours and minutes, portions of the extract were withdrawn, diluted 1:1 with Hoechst 33258 [10 μg/ml in 26% formaldehyde, 0.2 M sucrose, and 10 mM Hepes (pH 8.0)], and examined by fluorescence microscopy. **(C)** DNA replication was detected by pulse labeling of DNA in extracts with α^{32}P–deoxycytidine 5′-triphosphate, agarose gel electrophoresis, and autoradiography, as described (*22*).

Pin1 that compromised the prolyl isomerase activity of the enzyme by >90% (*30*) without diminishing its protein binding avidity (Fig. 4D). This mutant was incapable of restoring the checkpoint response in Pin1-depleted extracts when added to achieve concentrations sufficient for complementation of the checkpoint defect by wild-type xPin1 (Fig. 4B). Furthermore, *Xenopus* Pin1 complements the

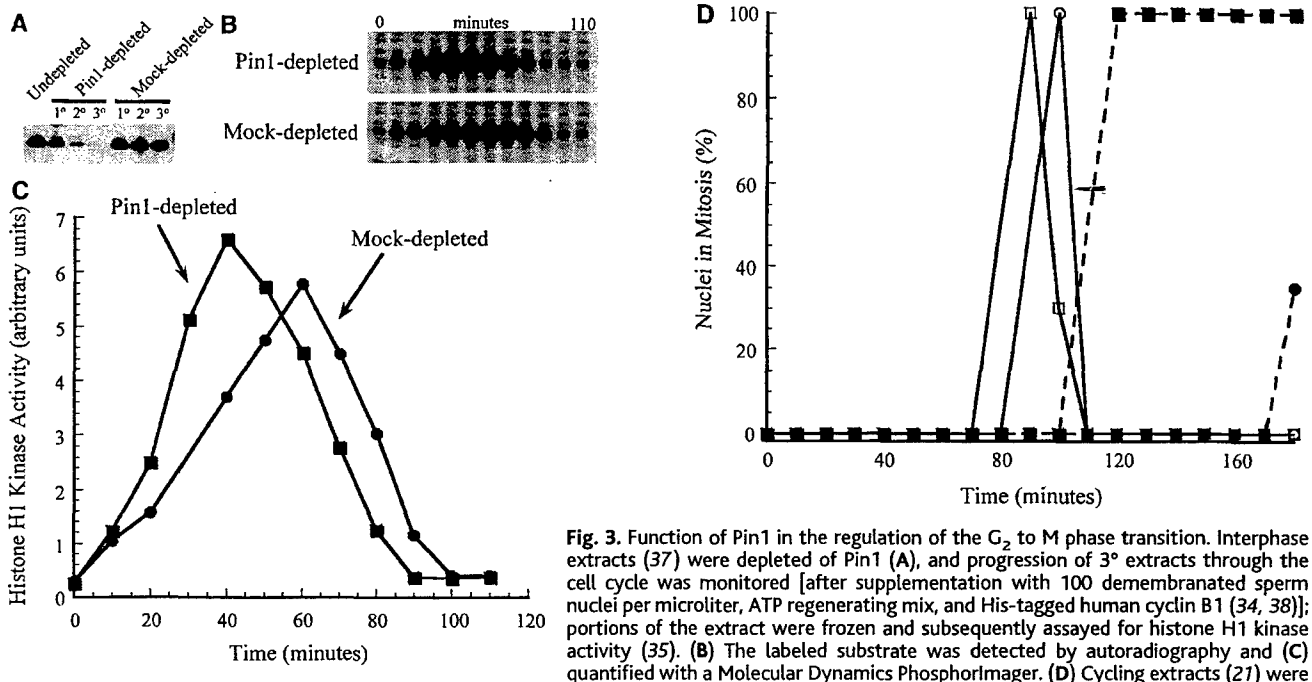

Fig. 3. Function of Pin1 in the regulation of the G_2 to M phase transition. Interphase extracts (*37*) were depleted of Pin1 (**A**), and progression of 3° extracts through the cell cycle was monitored [after supplementation with 100 demembranated sperm nuclei per microliter, ATP regenerating mix, and His-tagged human cyclin B1 (*34, 38*)]; portions of the extract were frozen and subsequently assayed for histone H1 kinase activity (*35*). (**B**) The labeled substrate was detected by autoradiography and (**C**) quantified with a Molecular Dynamics PhosphorImager. (**D**) Cycling extracts (*21*) were depleted of Pin1 (squares) or mock depleted (circles) and supplemented with ATP regenerating mix and 100 demembranated sperm nuclei per microliter (open symbols) or 500 nuclei per microliter (solid symbols). The mitotic index was monitored by fluorescence microscopy. Each of these experiments is representative of our observations in several extracts.

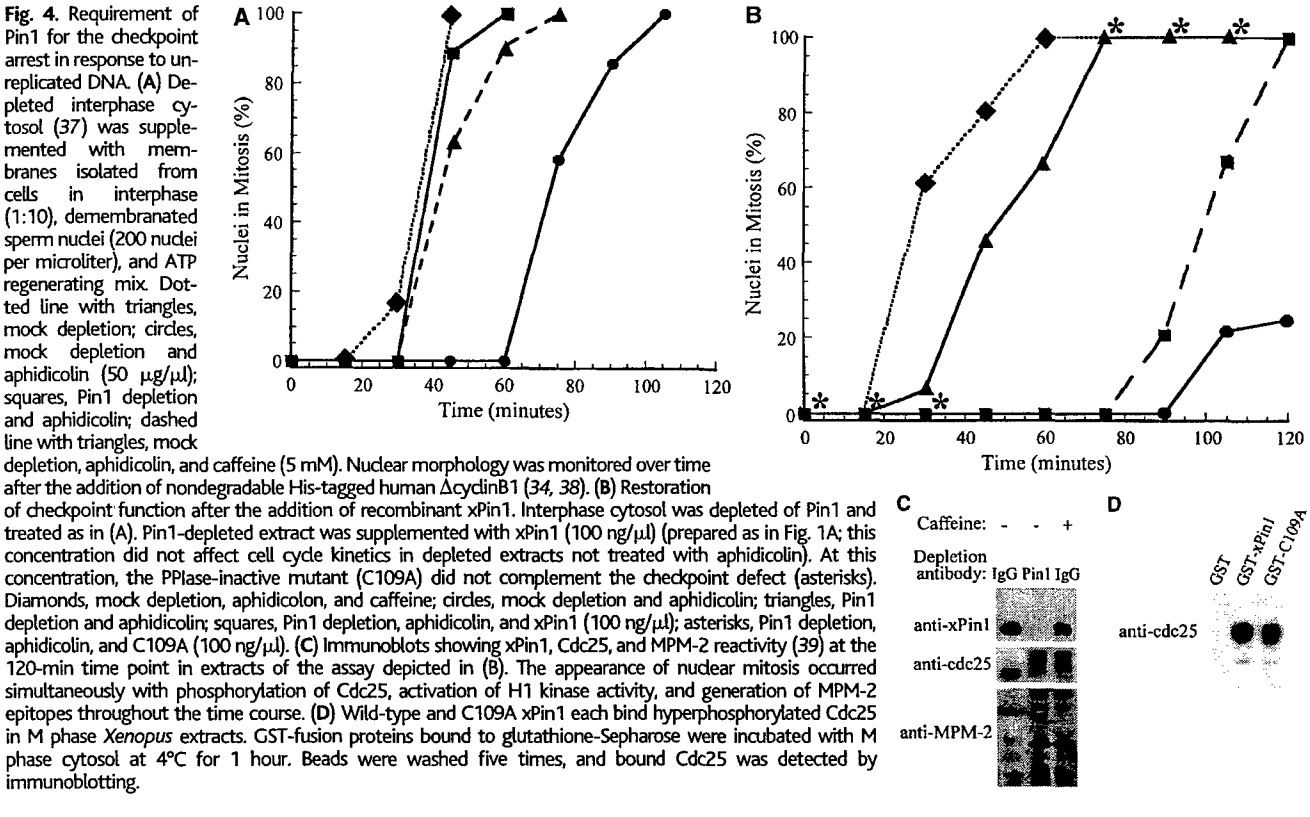

Fig. 4. Requirement of Pin1 for the checkpoint arrest in response to unreplicated DNA. (**A**) Depleted interphase cytosol (*37*) was supplemented with membranes isolated from cells in interphase (1:10), demembranated sperm nuclei (200 nuclei per microliter), and ATP regenerating mix. Dotted line with triangles, mock depletion; circles, mock depletion and aphidicolin (50 μg/μl); squares, Pin1 depletion and aphidicolin; dashed line with triangles, mock depletion, aphidicolin, and caffeine (5 mM). Nuclear morphology was monitored over time after the addition of nondegradable His-tagged human ΔcyclinB1 (*34, 38*). (**B**) Restoration of checkpoint function after the addition of recombinant xPin1. Interphase cytosol was depleted of Pin1 and treated as in (A). Pin1-depleted extract was supplemented with xPin1 (100 ng/μl) (prepared as in Fig. 1A; this concentration did not affect cell cycle kinetics in depleted extracts not treated with aphidicolin). At this concentration, the PPIase-inactive mutant (C109A) did not complement the checkpoint defect (asterisks). Diamonds, mock depletion, aphidicolon, and caffeine; circles, mock depletion and aphidicolin; triangles, Pin1 depletion and aphidicolin; squares, Pin1 depletion, aphidicolin, and xPin1 (100 ng/μl); asterisks, Pin1 depletion, aphidicolin, and C109A (100 ng/μl). (**C**) Immunoblots showing xPin1, Cdc25, and MPM-2 reactivity (*39*) at the 120-min time point in extracts of the assay depicted in (B). The appearance of nuclear mitosis occurred simultaneously with phosphorylation of Cdc25, activation of H1 kinase activity, and generation of MPM-2 epitopes throughout the time course. (**D**) Wild-type and C109A xPin1 each bind hyperphosphorylated Cdc25 in M phase *Xenopus* extracts. GST-fusion proteins bound to glutathione-Sepharose were incubated with M phase cytosol at 4°C for 1 hour. Beads were washed five times, and bound Cdc25 was detected by immunoblotting.

lethality of *ess1* mutants in budding yeast, but the C109A mutant is inactive in the complementation assay (*31*). Together, these observations indicate that Pin1 is functionally conserved and that its catalytic activity is required for its checkpoint role in *Xenopus* and its essential function in *S. cerevisiae*.

Pin1 participates in the replication checkpoint in a manner requiring its catalytic activity. Target-specific inhibition of mitosis-promoting kinase activity provides a biochemical mechanism for Pin1's role in enabling the replication checkpoint. Pin1 could mediate inhibition of Cdc25 hyperphosphorylation and act in concert with 14-3-3–mediated cytoplasmic sequestration of the phosphatase to prolong the G_2 phase by preventing functional interaction of Cdc25 with Cdc2 under checkpoint conditions. This function is consistent with previous observations that the G_2 phase is prolonged when Pin1 is overexpressed. In the absence of DNA perturbation, consequences of Pin1 removal may not be manifested; this would explain the lack of apparent phenotype in Pin1-null metazoans. Our demonstration of the essential role of Pin1 in the replication checkpoint establishes a position for endogenous Pin1 in the eukaryotic cell cycle regulatory network.

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Requirement of the Inositol Trisphosphate Receptor for Activation of Store-Operated Ca^{2+} Channels

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The coupling mechanism between endoplasmic reticulum (ER) calcium ion (Ca^{2+}) stores and plasma membrane (PM) store-operated channels (SOCs) is crucial to Ca^{2+} signaling but has eluded detection. SOCs may be functionally related to the TRP family of receptor-operated channels. Direct comparison of endogenous SOCs with stably expressed TRP3 channels in human embryonic kidney (HEK293) cells revealed that TRP3 channels differ in being store independent. However, condensed cortical F-actin prevented activation of both SOC and TRP3 channels, which suggests that ER-PM interactions underlie coupling of both channels. A cell-permeant inhibitor of inositol trisphosphate receptor ($InsP_3R$) function, 2-aminoethoxydiphenyl borate, prevented both receptor-induced TRP3 activation and store-induced SOC activation. It is concluded that $InsP_3Rs$ mediate both SOC and TRP channel opening and that the $InsP_3R$ is essential for maintaining coupling between store emptying and physiological activation of SOCs.

Receptor-induced Ca^{2+} signals comprise two interdependent components—rapid Ca^{2+} release from Ca^{2+} stores in the ER and Ca^{2+} entry through slowly activating PM SOCs. The trigger for SOC activation is decreased Ca^{2+} in the ER lumen (*1*, *2*). However, despite intense study, the ER-derived signal coupling store depletion with SOC activation remains unknown (*3*). Direct coupling between ER and PM has been hypothesized (*4*, *5*), and evidence indicates that physical docking of ER with the PM is involved in SOC activation (*6*–*8*). The mammalian TRP family of receptor-operated ion channels has been suggested to share some operational

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Maintenance of G_2 arrest in the *Xenopus* oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import

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Cdc2–cyclin B1 in the G_2-arrested *Xenopus* oocyte is held inactive by phosphorylation of Cdc2 at two negative regulatory sites, Thr14 and Tyr15. Upon treatment with progesterone, these sites are dephosphorylated by the dual specificity phosphatase, Cdc25, leading to Cdc2–cyclin B1 activation. Whereas maintenance of the G_2 arrest depends upon preventing Cdc25-induced Cdc2 dephosphorylation, the mechanisms responsible for keeping Cdc25 in check in these cells have not yet been described. Here we report that Cdc25 in the G_2-arrested oocyte is bound to 14-3-3 proteins and that progesterone treatment abrogates this binding. We demonstrate that Cdc25, apparently statically localized in the cytoplasm, is actually capable of shuttling in and out of the oocyte nucleus. Binding of 14-3-3 protein markedly reduces the nuclear import rate of Cdc25, allowing nuclear export mediated by a nuclear export sequence present in the N-terminus of Cdc25 to predominate. If 14-3-3 binding to Cdc25 is prevented while nuclear export is inhibited, the coordinate nuclear accumulation of Cdc25 and Cdc2–cyclin B1 facilitates their mutual activation, thereby promoting oocyte maturation.

Keywords: 14-3-3 protein/Cdc2–cyclin B1/Cdc25/oocyte maturation/*Xenopus*

Introduction

Xenopus oocytes are physiologically arrested in G_2 of meiosis I. Upon treatment with progesterone, these oocytes undergo meiotic maturation, leading to breakdown of the nuclear envelope (germinal vesicle breakdown; GVBD), chromosome condensation and spindle formation. Whereas many of the molecular details of this process have yet to be elucidated, it is clear that obligatory steps in progesterone-induced oocyte maturation include translation of mRNA encoding the mos protein kinase, consequent activation of a mitogen-activated protein kinase (MAPK) cascade and activation of maturation promoting factor (MPF), consisting of a Cdc2 kinase catalytic subunit complexed to a B-type cyclin (Masui and Markert, 1971; Sagata *et al.*, 1989; Kanki and Donoghue, 1991; Nebreda and Hunt, 1993; Shibuya and Ruderman, 1993; Kosako *et al.*, 1994).

In the oocyte, Cdc2–cyclin B complexes are stockpiled in an inactive form, poised for progesterone-induced activation. Phosphorylation of the Cdc2 subunit at two negative regulatory sites, Thr14 and Tyr15, is primarily responsible for the inactivity of the stored complexes (Cyert and Kirschner, 1988; Gautier and Maller, 1991; Kobayashi *et al.*, 1991). In somatic cells, phosphorylation of Tyr15 is catalyzed by two related kinases: a nuclear kinase called Wee1, and a cytoplasmic, membrane-associated kinase called Myt1 [Myt1 also phosphorylates Thr14; reviewed in Coleman and Dunphy (1994) and Lew and Kornbluth (1996)]. However, Wee1 appears to be entirely absent from *Xenopus* oocytes, so Myt1 is thought to be primarily responsible for phosphorylating Cdc2 in these cells (Murakami and Vande Woude, 1998). It has recently been reported that progesterone treatment leads to inactivation of Myt1, through a MAPK-induced activation of the kinase $p90^{rsk}$ (Palmer *et al.*, 1998). Myt1 physically associates with the active hyperphosphorylated form of rsk and phosphorylation of Myt1 by rsk inhibits Myt1 activity. Presumably, inactivation of Myt1 allows the dephosphorylation of Cdc2 Thr14 and Tyr15, leading to MPF activation (Atherton-Fessler *et al.*, 1994; Kornbluth *et al.*, 1994; Mueller *et al.*, 1995). Dephosphorylation of Thr14 and Tyr15 on Cdc2 is catalyzed by the dual (Thr/Tyr) specificity phosphatase, Cdc25 (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Strausfeld *et al.*, 1991; Millar and Russell, 1992). In somatic cells, Cdc25 alternates between an interphase form with low activity and a hyperphosphorylated mitotic form with high activity. Oocyte maturation is also accompanied by Cdc25 hyperphosphorylation (as evidenced by a shift in the electrophoretic mobility of Cdc25) and activation (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Hoffmann *et al.*, 1993).

Although physiologically distinct from the G_2 arrest of the oocyte, the checkpoint-induced G_2 arrest of somatic cells in response to DNA damage or stalled DNA replication also involves the suppression of pre-formed Cdc2–cyclin B complexes through phosphorylation of Cdc2 at Thr14 and Tyr15 (Enoch and Nurse, 1990; Enoch *et al.*, 1992; Smythe and Newport, 1992). Several groups have recently demonstrated that checkpoint-activated kinases phosphorylate Cdc25 at a critical regulatory site (Ser216 of human Cdc25C or Ser287 of *Xenopus* Cdc25; Peng *et al.*, 1997; Kumagai *et al.*, 1998a; Zeng *et al.*, 1998). This phosphorylation creates a binding site for members of a family of small acidic proteins collectively called 14-3-3 proteins (Peng *et al.*, 1997; Kumagai *et al.*, 1998b; Zeng *et al.*, 1998). Binding by 14-3-3 seems to functionally 'inactivate' Cdc25, and is critical for maintaining the checkpoint-induced G_2 arrest. Interestingly, 14-3-3 binding does not alter Cdc25 activity assayed *in vitro*, suggesting that 14-3-3 somehow sequesters Cdc25, perhaps altering its subcellular localization to prevent access of Cdc25 to the Cdc2–cyclin B substrates (Peng *et al.*, 1997).

Factors which modulate the subcellular localization of Cdc2–cyclin B complexes may also contribute to the maintenance of DNA-responsive checkpoint-induced cell-cycle arrest (Jin *et al.*, 1998; Toyoshima *et al.*, 1998). We and others have recently shown that Cdc2–cyclin B1 complexes, which appear to be statically localized to the cytoplasm during interphase, actually shuttle continuously in and out of the nucleus, where they might in some way 'read' the status of the DNA (Hagting *et al.*, 1998; Toyoshima *et al.*, 1998; Yang *et al.*, 1998). During interphase, nuclear export of cyclin B1, mediated by the nuclear export receptor CRM1, predominates over nuclear import. However, at the G_2/M transition, phosphorylation of cyclin B1 in the region of its nuclear export sequence (NES) prevents nuclear export, thereby fostering the nuclear accumulation of Cdc2–cyclin B1 required for the nuclear events of mitosis (Li *et al.*, 1995, 1997; Yang *et al.*, 1998). Although it has not been demonstrated that the DNA damage or replication checkpoints directly regulate cyclin B localization, there are data to suggest that this may be the case. Indeed, forcibly localizing cyclin B1 to the nucleus by appending a strong nuclear localization sequence, or by inactivating its nuclear export, compromises checkpoint function (Jin *et al.*, 1998; Toyoshima *et al.*, 1998).

In this report, we show that Cdc25 in the G_2-arrested *Xenopus* oocyte is complexed to 14-3-3 proteins, and that this binding is abrogated by progesterone treatment. Further, we demonstrate that the apparently cytoplasmic *Xenopus* Cdc25 contains an intrinsic CRM1-binding nuclear export sequence and can, like cyclin B1, shuttle in and out of the nucleus. Mutation of Cdc25 to prevent 14-3-3 binding resulted in a dramatic increase in the nuclear import rate of Cdc25, without markedly perturbing its nuclear export rate. These findings indicate that the G_2 arrest of the oocyte employs similar strategies to those operating in response to checkpoint controls, and provides a mechanistic basis for the functional inhibition of Cdc25 by 14-3-3 proteins.

Results

To determine whether *Xenopus* Cdc25 bound 14-3-3 proteins in the G_2-arrested oocyte, we prepared extracts from either untreated oocytes or oocytes after progesterone treatment. When anti-Cdc25 immunoprecipitates from these extracts were immunoblotted with antisera directed against 14-3-3 ε, the predominant Cdc25-binding variant in interphase *Xenopus* egg extracts (Kumagai *et al.*, 1998), we found that the G_2-arrested oocytes contained Cdc25–14-3-3 complexes, which were no longer detectable at the time of progesterone-induced GVBD (Figure 1). These data suggested the possibility that Cdc25 binding by 14-3-3 proteins might contribute to the maintenance of G_2 arrest in the oocyte.

Cytoplasmic accumulation of Cdc25 reflects continuous nuclear import and rapid re-export

Since binding of Cdc25 by 14-3-3 proteins does not appear to alter Cdc25 enzymatic activity, we wished to explore the possibility that 14-3-3 binding might regulate the subcellular localization of Cdc25 in the oocyte. Like cyclin B1, Cdc25C is cytoplasmic during interphase and enters

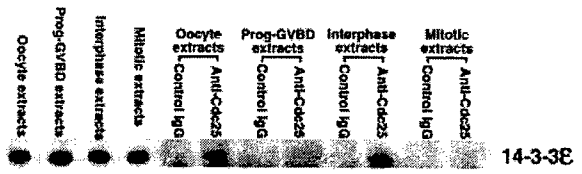

Fig. 1. *Xenopus* Cdc25 binds to 14-3-3 ε in oocyte extracts and interphase egg extracts. Extracts were prepared from untreated *Xenopus* oocytes, oocytes undergoing progesterone-induced GVBD, *Xenopus* eggs arrested in mitosis or *Xenopus* eggs released into interphase. Two microliters of each extract was subjected to SDS–PAGE and immunoblotted with anti-14-3-3 ε antibody (first four lanes). In addition, 60 μl of each extract was immunoprecipitated with either control IgG or anti-Cdc25 sera. Immunoprecipitated proteins were analyzed by SDS–PAGE followed by Western blotting with anti-14-3-3 ε antibody. 14-3-3 ε coprecipitated with Cdc25 only in the untreated oocyte and interphase egg extracts.

the nucleus just prior to mitosis, at least in some somatic cells (Seki *et al.*, 1992). Also like cyclin B1, Cdc25 is almost entirely cytoplasmic in the G_2-arrested *Xenopus* oocyte (Izumi *et al.*, 1992; Yang *et al.*, 1998). We have recently shown that cyclin B1 in fact shuttles in and out of the germinal vesicle (GV), leading us to suspect that the apparently static localization of Cdc25 belied its ability to shuttle in and out of nuclei. To test this possibility, we monitored Cdc25 localization after treating oocytes with leptomycin B, an inhibitor of CRM1-mediated nuclear export (Fornerod *et al.*, 1997; Neville *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997; Wolff *et al.*, 1997). Anti-Cdc25 immunoblotting of nuclear and cytoplasmic fractions from manually dissected oocytes revealed that inhibiting nuclear export promoted a striking nuclear accumulation of Cdc25, which occurred even faster than cyclin B1 nuclear accumulation in the same system (Figure 2A and B; Yang *et al.*, 1998). Thus, as for cyclin B1, cytoplasmic localization of Cdc25 results from ongoing nuclear import and more rapid re-export.

Xenopus Cdc25 is a nuclear export substrate in both the presence and absence of 14-3-3 binding

To determine if 14-3-3 binding affected Cdc25 localization, we wished to examine the consequences of this binding for Cdc25 nuclear import and export rates. To confirm that Cdc25 could, indeed, serve as a nuclear export substrate, we injected ^{35}S-labeled *in vitro*-translated Cdc25 into oocyte nuclei, and then manually dissected the oocytes into cytoplasmic and nuclear fractions at various times after injection. Within 60 min, virtually all of the Cdc25 had been exported from nuclei (Figure 2C). When we injected nuclei from the same batch of oocytes with Cdc25 protein which had been mutated to abrogate 14-3-3 binding (Cdc25 S287A), we found that the mutant Cdc25 appeared to exit nuclei at a rate only marginally slower than the wild-type (a point we will return to below).

Identification of a CRM1-binding NES in Cdc25

The inhibition of Cdc25 nuclear export by leptomycin-B suggested that Cdc25 export was mediated by CRM1. We produced recombinant derivatives of Cdc25 in *Escherichia coli* to examine whether they could bind to CRM1 in oocyte extracts. First, we fused only the N-terminal 322 amino acids or the C-terminal 228 amino acids of Cdc25 to glutathione *S*-transferase (GST), immobilized

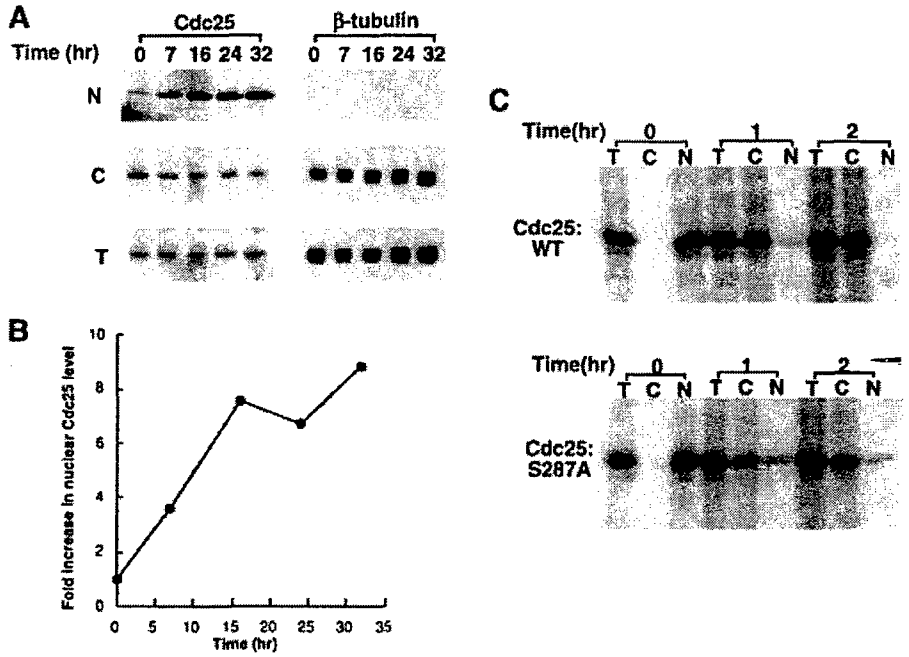

Fig. 2. Leptomycin B treatment induces endogenous Cdc25 nuclear accumulation in *Xenopus* oocytes and *Xenopus* Cdc25 can be exported from nuclei both in the presence and absence of 14-3-3 binding. (**A**) Oocytes were treated with 200 nM leptomycin B in MB buffer. At the times indicated after treatment, they were dissected into cytoplasmic and nuclear fractions. Proteins were extracted and analyzed by SDS–PAGE followed by immunoblotting with anti-*Xenopus* Cdc25 antibody. The samples were also blotted with anti-β-tubulin antibody as a control for dissection and loading. T, total; C, cytoplasmic fraction; N, nuclear fraction. For every oocyte nuclear equivalent loaded onto the gel, only 1/8 the amount of cytoplasm was loaded to facilitate observation of potential changes in cytoplasmic levels of the protein. For every oocyte nuclear equivalent loaded on the gel, 1/8 of a total oocyte was loaded in the 'T' lane. (**B**) The graph represents a quantitation of the data in (A), showing the fold increase of endogenous Cdc25 accumulated in nuclei at the times indicated after leptomycin B treatment. (**C**) Wild-type or S287A mutant Cdc25 protein was translated in reticulocyte lysates in the presence of ^{35}S-methionine and injected into *Xenopus* oocytes. At 0, 1 and 2 h after injection, the oocytes were dissected and successfully injected oocytes were identified by the presence of pink coloring from the reticulocyte lysate. These oocytes were separated into cytoplasmic and nuclear fractions, extracted and analyzed by SDS–PAGE followed by autoradiography.

the fusion proteins on glutathione–Sepharose and incubated these in oocyte extract as a source of CRM1. After extensive washing, proteins remaining bound to the beads were resolved by SDS–PAGE and immunoblotted with CRM1 antisera. As shown in Figure 3A, a protein containing the N-terminal 322 amino acids of Cdc25 was able to bind CRM1, whereas a protein containing the C-terminal 228 amino acids of Cdc25 was not. Furthermore, a Cdc25 N-terminal fragment containing the S287A mutation bound equally well to CRM1 despite its inability to bind 14-3-3 (Figure 3B). This suggests that the Cdc25–CRM1 interaction is unaffected by 14-3-3, consistent with the observed similar export rates of the full-length wild-type and S287A mutant Cdc25 proteins.

To localize the Cdc25 NES more precisely, we fused GST to successively smaller portions of the N-terminal fragment, serially deleting regions of the protein, starting from its C-terminus (aa 322; Figure 3C). As above, these fusion proteins were tested for their ability to retrieve CRM1 from oocyte extracts. We found that all of the fusion proteins examined, including one containing only the first 100 amino acids of Cdc25, were able to interact with CRM1 (Figure 3D). A scan of the first 100 amino acids revealed a single sequence, 47LTPVTDLAV55, matching the consensus sequence for a CRM1-binding, leucine-rich NES (Bogerd *et al.*, 1996). To determine whether this sequence affected nuclear export of Cdc25, we mutated the last two required hydrophobic residues, L53 and V55, to Ala (Figure 4A). This mutant protein was not detectably exported following microinjection into oocyte nuclei (Figure 4B and C) and a recombinant truncated Cdc25 protein bearing the same mutations bound well to 14-3-3 protein, but could not bind to CRM1 (Figure 4D). These data strongly suggest that the mutated residues lie within a functional NES which is responsible for *Xenopus* Cdc25 nuclear export.

Cdc25 S287A induces GVBD in leptomycin-B-treated oocytes

Since 14-3-3 binding did not appear to modulate the nuclear export rate of Cdc25, we wished to determine whether the nuclear import rate of Cdc25 might be affected. To this end, we injected radiolabeled wild-type or S287A Cdc25 proteins into the cytoplasm of oocytes which had been pre-treated with leptomycin B to prevent re-export. Intriguingly, under these conditions the S287A protein induced MPF activation (assayed as histone H1-directed kinase activity) and GVBD ~6 h after injection (Figure 5A). This effect required leptomycin B treatment and did not occur with the wild-type Cdc25. Since we injected trace quantities of radiolabeled protein (<2% of endogenous Cdc25), this result indicates that the S287A Cdc25 mutant has considerably increased biological potency compared with the wild-type, in agreement with the recently described relative potency of similar Cdc25 mutants in overcoming a checkpoint arrest in somatic

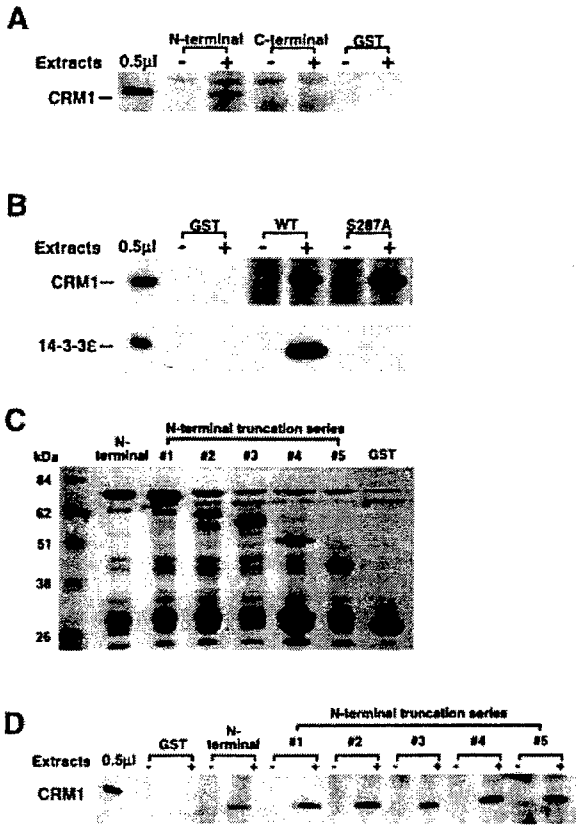

Fig. 3. The N-terminal region of *Xenopus* Cdc25 contains a CRM1-binding NES, which can bind to CRM1 in the presence and absence of 14-3-3 binding. **(A)** N-terminal (aa. 1–322) or C-terminal (aa. 323–550) fragments of Cdc25 were fused to GST and coupled to glutathione–Sepharose beads. Twenty microliters of these resins or control GST resin were incubated in 100 µl of interphase extract for 1 h. The beads were then pelleted and washed five times with egg lysis buffer. The bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-human CRM1 antibody. **(B)** The N-terminal (aa 1–322) fragments of wild-type or S287A mutant Cdc25 proteins were fused to GST and coupled to glutathione–Sepharose beads. The pull-down assay was performed as in (A), and the bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-CRM1 and anti-14-3-3 ε antibody. **(C)** The truncated proteins derived from the N-terminal fragment of Cdc25 were fused to GST and coupled to glutathione–Sepharose beads. The bead-bound proteins were boiled in SDS sample buffer and eluted proteins were subjected to SDS–PAGE and Coomassie-blue staining, to show the molecular weights of the deleted fusion proteins. **(D)** Twenty microliters of the resins shown in (C) were used for pull-down assays as described in (A). The bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-human CRM1 antibody.

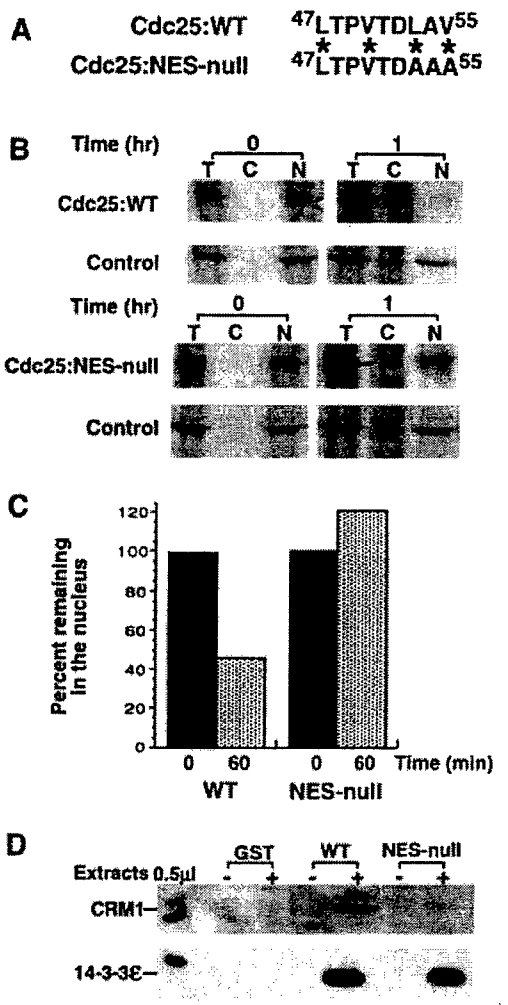

Fig. 4. Identification of the CRM1-binding NES in *Xenopus* Cdc25. **(A)** Residues 47–55 of *Xenopus* Cdc25 comprise a putative NES. Asterisks indicate consensus leucine-rich residues. L53 and V55 were mutated to Ala to create the Cdc25:NES-null protein. **(B)** ^{35}S-labeled Cdc25:WT or Cdc25:NES-null proteins were coinjected into oocyte nuclei with GRP94 control protein. Oocytes were dissected 0 or 1 h later and proteins were extracted and analyzed by SDS–PAGE and autoradiography. By 2 h (not shown), the NES-null protein had still not exited from nuclei. **(C)** The bar graph represents a quantitation of the data in (B), showing the percentage of Cdc25 remaining in nuclei after 0 and 1 h. Values were normalized to coinjected GRP94 protein. **(D)** The N-terminal (aa. 1–322) fragment of wild-type or NES-null mutant Cdc25 proteins were fused to GST and coupled to glutathione–Sepharose beads. The pull-down assay was performed as in Figure 3A, and the bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-CRM1 and anti-14-3-3 ε antibody.

cells (Kumagai *et al.*, 1998b; Peng *et al.*, 1997; Zeng *et al.*, 1998).

Intra-nuclear activation of Cdc25 S287A by Cdc2–cyclin B

In the above experiments, GVBD was preceded by nuclear accumulation and hyperphosphorylation of the S287A Cdc25, detected by an electrophoretic mobility shift upon SDS–PAGE (Figure 5B). A similar mobility shift of Cdc25 has been reported in response to phosphorylation by active Cdc2–cyclin B and by the kinase Plx1 (Kumagai and Dunphy, 1992, 1996; Hoffmann *et al.*, 1993; Izumi and Maller, 1995). In both cases, Cdc25 hyperphosphorylation correlates with an increase in its enzymatic activity. Since leptomycin B treatment also induces nuclear accumulation of Cdc2–cyclin B1, we wished to determine whether Cdc2–cyclin B might be responsible for the hyperphosphorylation of S287A Cdc25 in leptomycin-B-treated oocytes. Therefore, we repeated the wild-type and mutant Cdc25 injection experiments using oocytes which had been pre-incubated with both leptomycin B and the Cdc2 inhibitor, roscovitine (50 µM; Meijer *et al.*, 1997). Roscovitine effectively eliminated the S287A Cdc25 mobility shift (and GVBD), consistent with a role for

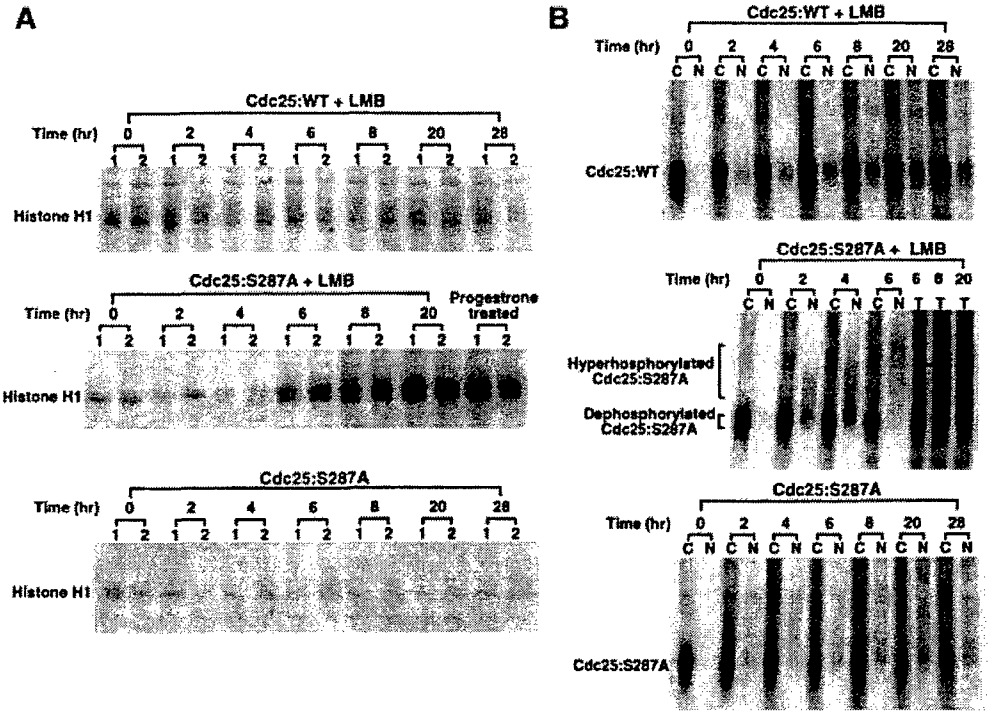

Fig. 5. Cdc25:S287A protein induces GVBD in leptomycin B-treated oocytes and is hyperphosphorylated in these oocyte nuclei. (**A**) Oocytes were incubated with or without 200 nM leptomycin B in MB buffer for 2 h before injection. Forty nanoliters of ^{35}S-labeled Cdc25:WT or S287A protein in reticulocyte lysate was coinjected into oocyte cytoplasm with ^{14}C-labeled BSA as control. Two oocytes were collected at each of the indicated times after injection and immediately frozen in liquid nitrogen. The samples were assayed for Cdc2 kinase activity using histone H1 as a substrate. Progesterone-treated oocytes, which had already undergone GVBD, were used as a positive control. (**B**) Oocytes treated and injected as described in (A), oocytes were dissected into cytoplasmic and nuclear fractions at the indicated times after injection and proteins were analyzed by SDS–PAGE and autoradiography. For leptomycin-B-treated oocytes injected with S287A Cdc25, oocytes started GVBD at 6 h and had all undergone GVBD by 8 h. For oocytes injected with wild-type Cdc25 or not treated with leptomycin B, GVBD had not occurred even 28 h after injection.

Cdc2–cyclin B in the observed Cdc25 hyperphosphorylation (Figure 6A). At similar concentrations, roscovitine did not prevent Cdc25 hyperphosphorylation by kinases active in interphase egg extracts treated with the phosphatase inhibitor, microcystin (5 μM; Figure 6B). These egg extracts lack cyclins A and B (and hence lack active Cdc2), but are induced to enter a pseudomitotic state by incubation with microcystin and contain a variety of activated non-Cdc2 kinases including Plx1 (Izumi and Maller, 1995; Kumagai and Dunphy, 1996; Qian *et al.*, 1998). Thus, the lack of inhibition of these non-Cdc2 kinases by roscovitine supports its reported specificity as an inhibitor and suggests that Cdc2–cyclin B was, indeed, responsible for phosphorylating and activating S287A Cdc25 in the oocyte. In aggregate, these data suggest that abrogating the Cdc25–14-3-3 interaction with the S287A mutation creates a more potent Cdc25 that triggers a feedback loop involving the mutual activation of Cdc25 and Cdc2/cyclin-B in the nuclei of leptomycin-B-treated oocytes.

The S287A mutant of Cdc25 is imported into nuclei more efficiently than the wild-type protein

What is the basis for the increased potency of S287A Cdc25? Since both Cdc2–cyclin B1 and Cdc25 gradually accumulate in oocyte nuclei upon inhibition of nuclear export with leptomycin B, one possibility is that the S287A mutation increases the nuclear import rate of Cdc25. Faster accumulation of the trace amount of injected Cdc25 in the nucleus (away from the countervailing inhibitory action of Myt1 on Cdc2–cyclin B) may allow it to trigger the activating feedback loop leading to GVBD. To compare the nuclear import rates of wild-type and S287A Cdc25 proteins, we repeated the cytoplasmic injection experiments, but used roscovitine to prevent GVBD and permit analysis of nuclear import (in leptomycin-B-treated oocytes, to prevent Cdc25 re-export). At various times after injection, oocytes were separated into cytoplasmic and nuclear fractions and resolved by SDS–PAGE. As shown in Figure 7, the S287A mutant protein accumulated in nuclei at a markedly faster rate than the wild-type protein. These data strongly suggest that S287 phosphorylation, and consequent 14-3-3 binding, reduces Cdc25 nuclear import in the oocyte.

How does 14-3-3 binding affect nuclear import of Cdc25? Scanning of the *Xenopus* Cdc25 sequence revealed an evolutionarily conserved, consensus bipartite basic NLS, KR X_{13} KRRR at amino acids 298–316. Consistent with its containing a classical NLS, the N-terminal 322 amino acids of Cdc25 bound to the nuclear import receptor for such sequences, importin-α/β (Figure 8A; Gorlich *et al.*, 1994, 1995). Mutation of residues 313–315 of the candidate NLS to Ala severely impaired nuclear import of the mutant Cdc25 (Figure 8B) and greatly reduced binding to the importin-α/β heterodimer (Figure 8A). Consistent with its enhanced rate of nuclear import, the S287A Cdc25 mutant bound significantly better than the wild-type Cdc25 protein to the importin-α/β heterodimer (Figure 9A and B).

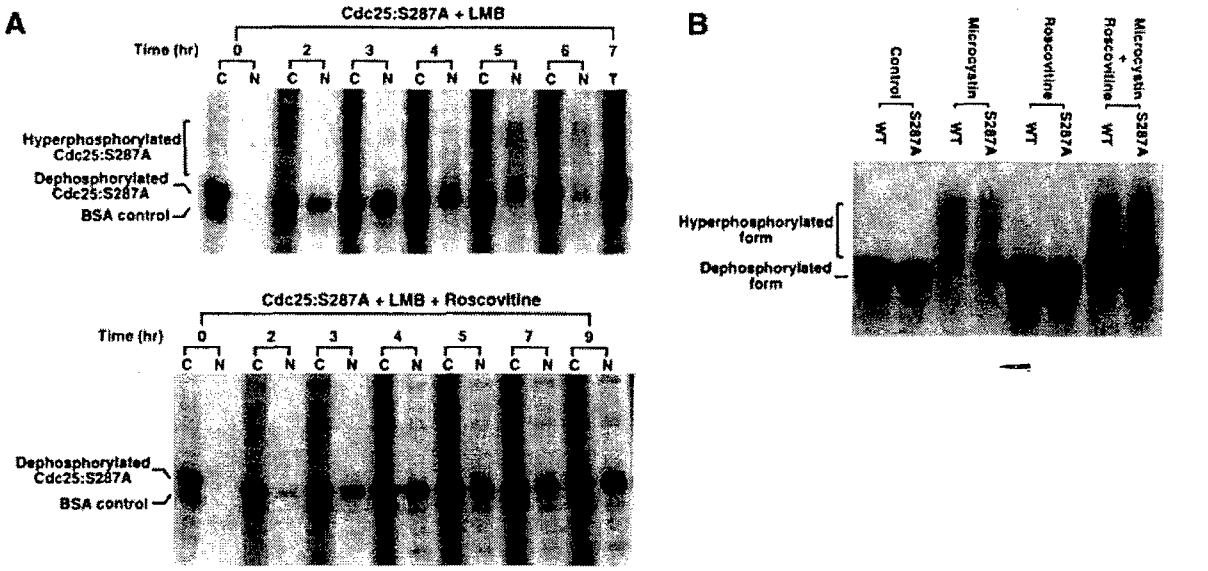

Fig. 6. Cdc2–cyclin B is responsible for the hyperphosphorylation of Cdc25:S287A in leptomycin-B-treated oocyte nuclei. **(A)** Oocytes were incubated with either 200 nM leptomycin B in MB buffer for 2 h before injection, or with both 200 nM leptomycin B and 50 μM roscovitine. Forty nanoliters of ^{35}S-labeled S287A protein translated *in vitro* was coinjected into oocyte cytoplasm with ^{14}C-labeled BSA as control. Oocytes were dissected into cytoplasmic and nuclear fractions at the indicated times after injection and proteins were analyzed by SDS–PAGE followed by autoradiography. Oocytes not treated with roscovitine entered GVBD at 7 h, whereas those treated with roscovitine did not manifest GVBD even after incubation overnight. **(B)** Two microliters of ^{35}S-labeled wild-type or S287A mutant Cdc25 protein was added to 20 μl of interphase *Xenopus* egg extract. These extracts were incubated with 5 μM microcystine, 50 μM roscovitine or 5 μM microcystin and 50 μM roscovitine at room temperature for 30 min in the presence of 2 mM ATP. The samples were subjected to SDS–PAGE and autoradiography.

When we injected NLS-null Cdc25 or S287A, NLS-null doubly mutant Cdc25 into oocyte nuclei to compare wild-type and S287A export rates under conditions where re-import could not occur, we did not observe any significant differences in their rates of nuclear export (Figure 9C). This demonstrates that the slight difference in the export rates of wild-type and S287A Cdc25 proteins shown in Figure 2C was due to faster re-import of exported S287A Cdc25. These data strongly suggest that 14-3-3 binding to *Xenopus* Cdc25 in oocytes exerts effects on nuclear shuttling by altering the rate of Cdc25 nuclear import, rather than export. Moreover, they indicate that 14-3-3 binding controls Cdc25 biological activity, at least in part, by inhibiting its entry into the nucleus.

Discussion

Binding of 14-3-3 to Cdc25 in G_2-arrested oocytes

We have shown that Cdc25 in the G_2-arrested oocyte can be found in a complex with 14-3-3 proteins, and that this complex is dissociated following progesterone treatment. In somatic cells, Cdc25 is phosphorylated in response to DNA damage and DNA-replication-induced checkpoint activation (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997). Recent studies have demonstrated that this phosphorylation (at residue S287 of *Xenopus* Cdc25) results in the formation of a 14-3-3–Cdc25 complex which is important for maintaining the checkpoint-induced G_2 arrest (Peng *et al.*, 1997; Kumagai *et al.*, 1998b; Zeng *et al.*, 1998). In *Xenopus* oocytes, S287 phosphorylation is also required for 14-3-3 binding. These parallels suggest that similar strategies are employed to maintain a G_2 arrest in oocytes and somatic cells in response to different physiological stimuli. It will be interesting to determine whether other pathways functioning in the oocyte, such as regulation of Myt1 activity by rsk, also contribute to mitotic control in somatic cells.

14-3-3 binding selectively inhibits nuclear import of Cdc25 in oocytes

The mechanism whereby 14-3-3 binding functionally suppresses Cdc25 activity has been mysterious. We have found that Cdc25 accumulates almost exclusively in the cytoplasm of the oocyte as a result of a steady-state situation in which Cdc25 slowly enters the nucleus and is rapidly re-exported back to the cytoplasm. Our data demonstrate that the rate of Cdc25 nuclear import is greatly accelerated by mutation of S287 to non-phosphorylatable Ala. This suggests that S287 phosphorylation, and consequent 14-3-3 binding, significantly reduce Cdc25 nuclear import. We have identified a functional and evolutionarily conserved NLS in Cdc25 that lies adjacent to the site of 14-3-3 binding. Hence, 14-3-3 binding may sterically block access of Cdc25 to the nuclear import machinery.

Export of Cdc25 from oocyte nuclei was inhibited by leptomycin B, suggesting the involvement of the export factor CRM1. Indeed, we found that an N-terminal fragment of Cdc25 containing a putative NES sequence could bind to CRM1. Mutagenesis experiments demonstrated that this sequence was critical for both CRM1 binding and for nuclear export of Cdc25, and therefore constituted a functional NES. Unlike its dramatic effect on the nuclear import of Cdc25, mutation of S287 to Ala had no significant effect on the rate of Cdc25 nuclear export. This was confirmed in export assays in which re-import of Cdc25 was eliminated by mutation of the Cdc25 NLS. Thus, binding of 14-3-3 selectively reduces the rate of

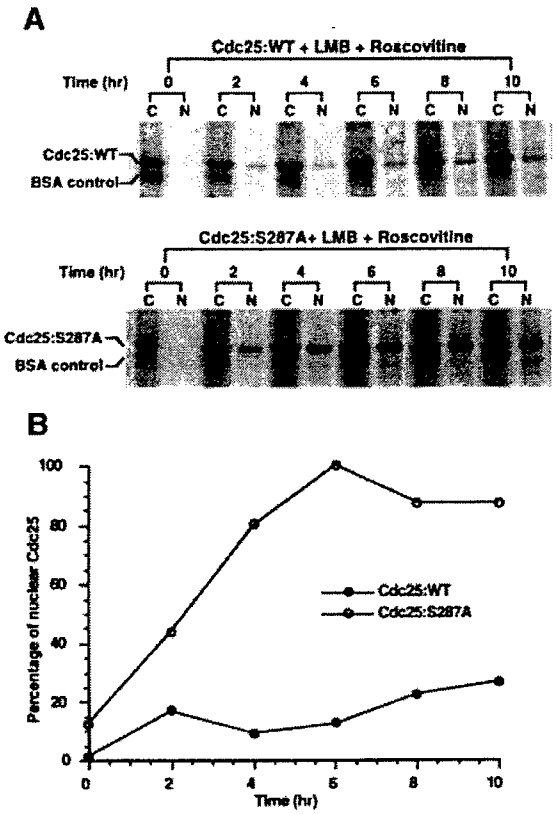

Fig. 7. The S287A mutant of Cdc25 is imported into nuclei more efficiently than the wild-type protein. **(A)** Oocytes were incubated with 200 nM leptomycin B and 50 μM roscovitine in MB buffer for 2 h before injection. Forty nanoliters of ^{35}S-labeled *in vitro* translated Cdc25:WT or S287A protein was injected into the cytoplasm of oocytes along with ^{14}C-labeled BSA as control. Injected oocytes were dissected into cytoplasmic and nuclear fractions at the indicated times after injection and proteins were analyzed by SDS–PAGE and autoradiography. **(B)** The graph represents a quantitation of the data in (A), showing the percentage of Cdc25 in the nuclear fraction at the indicated times.

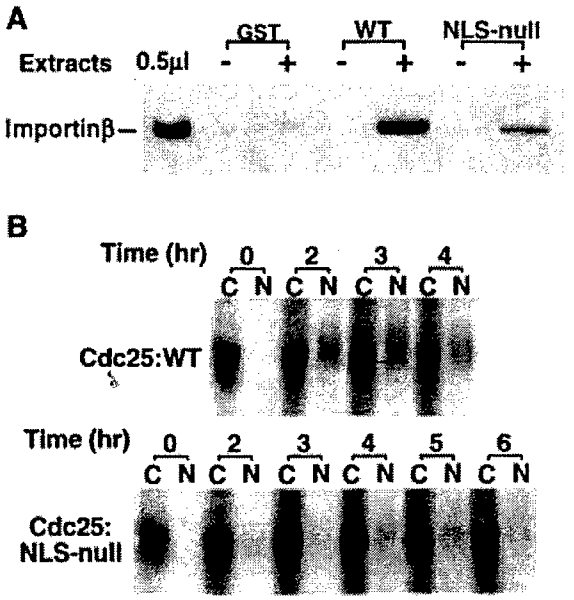

Fig. 8. Identification of a bipartite basic NLS in Cdc25. **(A)** The N-terminal (aa. 1–322) fragment of wild-type or NLS-null (K313A,R314A,R315A) mutant Cdc25 proteins were fused to GST and coupled to glutathione–Sepharose beads. Twenty microliters of these resins or control GST resin were incubated in 100 μl of interphase egg extract for 1 h. The beads were then pelleted and washed five times with egg lysis buffer. The bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-importin-β antibody. Note that classical NLSs bind importin-β via importin-α provided by the extract. **(B)** Forty nanoliters of ^{35}S-labeled *in vitro*-translated Cdc25:WT or Cdc25:NLS-null proteins were injected into the cytoplasm of oocytes. Injected oocytes were dissected into cytoplasmic and nuclear fractions at the times indicated after injection and proteins were analyzed by SDS–PAGE followed by autoradiography.

Cdc25 nuclear import while leaving its rate of export unaffected, presumably resulting in more efficient exclusion of Cdc25 from the nucleus.

Very recently, Lopez-Girona *et al.* (1999) reported studies in the fission yeast *Schizosaccharomyces pombe* which also indicate that 14-3-3 binding leads to exclusion of Cdc25 from the nucleus. However, this similar outcome was proposed to arise from a distinct mechanism, in which 14-3-3 binding provided a portable NES for Cdc25 nuclear export. This is clearly not the case in *Xenopus* oocytes, because non-phosphorylatable Cdc25 incapable of binding 14-3-3 is fully competent for export mediated by the Cdc25-intrinsic NES. Furthermore, a mutant Cdc25 lacking a functional NES was unable to be exported despite its continued ability to bind 14-3-3, suggesting that in this system 14-3-3 binding is neither necessary nor sufficient for Cdc25 nuclear export. These apparent differences between fission yeast and *Xenopus* may reflect the fact that the NES we have identified in *Xenopus* Cdc25 does not appear to be evolutionarily conserved. Thus, different cells may use different mechanisms for achieving nuclear exclusion of Cdc25 in response to 14-3-3 binding.

14-3-3 binding to Cdc25 collaborates with CRM1-mediated nuclear export of unknown factors to maintain the G_2 arrest in the oocyte

The importance of 14-3-3 binding for the suppression of Cdc25 biological activity was evident in experiments in which trace amounts of S287A Cdc25 injected into oocyte cytoplasm were able to induce GVBD, whereas similar amounts of wild-type Cdc25 were not. However, GVBD was only induced in oocytes treated with leptomycin B to inhibit CRM1-mediated nuclear export. A simple hypothesis to explain this requirement would be that oocyte maturation required retention of the imported Cdc25 in the nucleus. We tested this by injecting trace amounts of a doubly mutant Cdc25 lacking both a functional NES and the ability to bind 14-3-3. Although this protein was efficiently imported into and retained in oocyte nuclei, it did not promote GVBD unless oocytes were also treated with leptomycin B (data not shown). This suggests that factors other than Cdc25 must be retained in the nucleus to collaborate with the S287A Cdc25.

It has been reported that 14-3-3 binding to phosphorylated Cdc25 does not greatly affect its activity *in vitro*, producing a <2-fold reduction in activity (Peng *et al.*, 1997; Kumagai *et al.*, 1998b). However, even a slight increase in Cdc25 enzymatic activity resulting from the G_2/M loss of 14-3-3 binding might be sufficient, after concentration in the nucleus, to activate a small amount

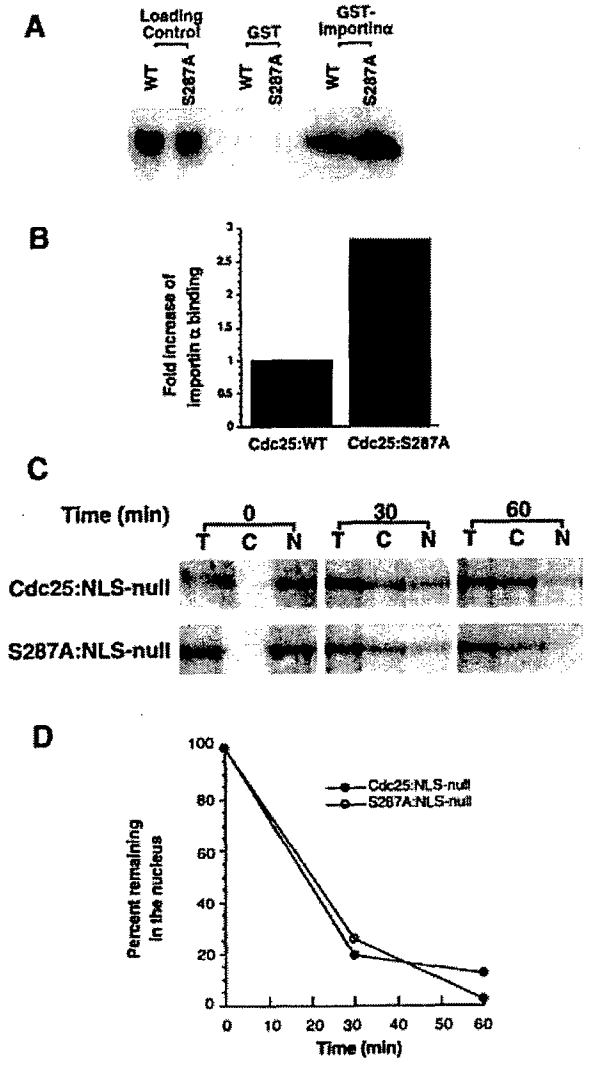

Fig. 9. The Cdc25:S287A mutant binds significantly better than the wild-type protein to importin-α/β, and import-defective variants of these proteins export from nuclei at a similar rate. (**A**) *Xenopus* importin-α protein was fused to GST and coupled to glutathione–Sepharose beads. Twenty microliters of this resin or control GST resin was incubated in 100 μl of interphase egg extract with 10 μl ^{35}S-labeled wild-type or S287A Cdc25 protein for 1 h. The beads were then pelleted and washed five times with egg lysis buffer. The bead-bound proteins were analyzed by SDS–PAGE followed by autoradiography. (**B**) The bar graph represents a quantitation of the data in (A), comparing the amounts of Cdc25:WT or Cdc25:S287A bound to importin-α/β (importin-α beads will associate with importin-β in the extract). (**C**) Cdc25:NLS-null or S287A:NLS-null protein was translated in reticulocyte lysates in the presence of [^{35}S] methionine and injected into *Xenopus* oocyte nuclei. At 0, 30 and 60 min after injection, the oocytes were dissected and successfully injected oocytes were identified by the presence of pink coloring from the reticulocyte lysate. These oocytes were separated into cytoplasmic and nuclear fractions, extracted and analyzed by SDS–PAGE followed by autoradiography. (**D**) The graph represents a quantitation of the data in (C), showing the percentage of Cdc25 remaining in nuclei after 0, 30 and 60 min.

of nuclear Cdc2–cyclin B; this would effectively set in motion a positive feedback loop. In the absence of bound 14-3-3 protein, Cdc25 may also be more susceptible to activation, either by Cdc2–cyclin B (in a feedback loop) or by other nuclear Cdc25-activating kinases. Reconstitution of the Cdc2–cyclin B–Cdc25 positive feedback loop with fully purified components will be required to distinguish between these possibilities. None the less, it is attractive to speculate that Cdc2–cyclin B is the factor which must be concentrated in the nucleus along with Cdc25 to promote passage through the G_2/M transition. Once separated from the cytoplasmic Myt1 (and given the absence of nuclear Wee1 in oocytes), nuclear Cdc25 and Cdc2–cyclin B could very effectively activate each other through positive feedback mechanisms.

Materials and methods

Oocyte preparation, microinjection and subcellular fractionation

Stage VI oocytes of *Xenopus laevis* were prepared for microinjection, dissection and subcellular fractionation as described previously (Yang *et al.*, 1998). Two injection controls for nuclear integrity were used: ^{14}C-labeled bovine serum albumin (BSA; Amersham) and *in vitro*-translated ^{35}S-labeled GRP94 (a protein that does not have an NES or NLS). At each timepoint, five to 10 oocytes were collected for subfractionation and analyzed by SDS–PAGE (National Diagnostics protogel), followed by autoradiography or Western blotting.

Preparation of Xenopus oocyte and egg extracts

Oocyte extracts were prepared as described previously (Shibuya *et al.*, 1992). To induce GVBD, oocytes were treated with 5 μg/ml progesterone in modified Barth's (MB) buffer (Swenson *et al.*, 1989) overnight. Once GVBD was observed in the majority of oocytes, they were collected to make GVBD extracts; these extracts were prepared in mitotic egg extract buffer (Smythe and Newport, 1991) to preserve meiotic phosphorylations. Interphase egg extracts and mitotic extracts were prepared according to the protocols of Smythe and Newport (1991).

Coimmunoprecipitation experiments

Various extracts were incubated with the relevant sera at 4°C for 1 h. Protein A–Sepharose beads (Sigma, St Louis, MO) were then washed with extract buffer and incubated with the above extracts at 4°C for 2 h. Beads were pelleted and washed five times with the relevant extract buffer. Bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting.

In vitro translation

Xenopus Cdc25 wild-type, S287A, NLS-null and NES-null mutants were subcloned downstream of an SP6 promoter in the vector SP64T. ^{35}S-labeled proteins were produced using the SP6-coupled TNT reticulocyte system (Promega) according to the manufacturer's instructions.

Construction of the GST-N-terminal and C-terminal Cdc25 fusion proteins and the S287A Cdc25 mutant

To construct the N-terminal fragment of Cdc25, a stop codon was inserted after amino acid 322 by polymerase chain reaction (PCR) and the resulting clone was inserted into pGexKG through *Nco*I and *Xho*I sites incorporated into the oligonucleotides used for PCR. For production of the C-terminal fragment of Cdc25, amino acids 323–550 of Cdc25 were isolated by PCR and cloned into pGexkG through the *Nco*I and *Xba*I sites.

To produce the S287A mutant clone, a *Xenopus* cDNA clone kindly provided by Dr J.Maller (cdc25C1) was used as the template. The mutant was generated using the method described by Kunkel and colleagues (Kunkel, 1985; Kunkel *et al.*, 1987). Briefly, the mutant primer, 5′CCTTTACCGCTCACCTGCTATGCCAGAGAAAC3′ was annealed to a single-stranded pBluescriptSK$^+$–cdc25C1 DNA and the complementary strand was synthesized *in vitro*.

Construction of the Cdc25 N-terminal truncation series

The Erase-a-Base system (Promega) was used to generate a deletion series from amino acid 322 towards the N-terminus of Cdc25 in pGexKG. The DNA encoding the N-terminal fragment of Cdc25 in pGexKG was cut at its 3′ end with *Xho*I to generate a 5′ overhang for Exonuclease III digestion. It was also digested with *Sac*I to generate an adjacent 3′ overhang to protect the plasmid vector from Exonuclease III digestion. Exonuclease III deletion, ligation and transformation protocols were as

described by the manufacturer. Fifty clones were induced for GST-fusion protein expression. Based on their sizes, five clones were selected for binding assays.

Site-directed mutagenesis

For construction of the NES-null mutant Cdc25, PCR-based mutagenesis was used to mutate both Leu53 and Val55 to Ala. Wild-type *Xenopus* Cdc25 cDNA in the pSP64T vector was used as the template. The 5′ oligonucleotide encoding the N-terminus of Cdc25 was 5′-AATAGTG-AAGCCATGGCAGAGAGTCACATA-3′, where an *Nco*I site was inserted before the start codon. The 3′ oligonucleotide encoding the C-terminus of Cdc25 was 5′-GCGGCGGCCTCGAGATTAAAGCTT-CATCAGGCG-3′, where an *Xho*I site was inserted after the stop codon. The mutagenic PCR primers were 5′-TTGACACCTGTGACTGACGC-TGCAGCTGGATTTAGTAACCTAAGTAC-3′ and its reverse primer. Using the 5′ primer along with one mutagenic primer, we produced a PCR fragment extending from the 5′ end (encoding the extreme N-terminus) to the site of mutation. We then generated a second PCR product using the C-terminal primer and the second mutagenic primer, producing a DNA fragment extending from the mutation site to the C-terminal end. A full-length mutant clone was generated with an additional round of PCR, using a mixture of the N- and C-terminal encoding DNA fragments as templates for PCR with the original 5′ and 3′ primers. The full-length product was subcloned into the pSP64T vector. For construction of the NLS-null mutant Cdc25, the same strategy was used to mutate Lys313, Arg314 and Arg315 to Ala in order to destroy the consensus bipartite NLS. The PCR primers containing mutations were 5′-GAAACACCTGTCAGAGTGGCCGCGGCACGTA-GTACCAGCAGCCCC-3′ and its reverse primer. All mutations were confirmed by DNA sequencing.

Expression and purification of recombinant GST-fusion proteins

All constructs were expressed in Topp 3 *E.coli* (Stratagene). To increase solubility of recombinant proteins, bacteria were grown to OD = 0.5 at 37°C and then shifted to 18°C. Isopropyl β-D-thiogalactoside (0.4 mM) was added to induce protein expression at 18°C overnight. Bacteria were pelleted and resuspended in lysis buffer [50 mM Tris7.5, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. 1% Triton X-100 and 300 mM NaCl were added to increase protein solubility. Cells were lysed twice using a French Press and spun at 17 000 *g* for 30 min. The supernatants were diluted 1:1 with buffer (10 mM HEPES pH 8.0 and 1 mM DTT) to reduce the Triton concentration and incubated with glutathione–Sepharose beads at 4°C for 1–2 h. Beads were pelleted, washed with buffer (10 mM HEPES, pH 8.0, 300 mM NaCl and 1 mM DTT) and kept in storage buffer (10 mM HEPES pH 8.0, 50% glycerol, 1 mM DTT) at –20°C.

Pull-down experiments

GST fusion proteins were coupled to glutathione–Sepharose beads and washed with the appropriate extract buffer. The beads were then incubated with either different extracts or with extracts containing ^{35}S-labeled proteins at 4°C for 1–2 h. The beads were washed five times with the proper extract buffer and the binding proteins were resolved by SDS–PAGE followed by immunoblotting or autoradiography.

Histone H1 kinase assay of single oocytes

Single oocytes were thawed in 20 µl of oocyte lysis buffer (20 mM HEPES 7.5, 80 mM β-glycerolphosphate, 15 mM $MgCl_2$, 20 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 10 µg/ml aprotin/leupeptin, 1 mM PMSF) and lysed by pipetting. The sample was then microfuged at 4°C for 5 min and the supernatant was collected. To start the kinase reaction, 10 µl of the supernatant was incubated with 10 µl kinase buffer (20 mM HEPES 7.3, 10 mM EGTA, 20 mM $MgCl_2$, 10 µM protein kinase inhibitor (PKI), 0.2 mg/ml histone H1, 0.2 mM ATP and 0.5 µCi/µl [γ-^{32}P]ATP) at room temperature for 10 min. The reaction was stopped by addition of 20 µl of 2× SDS sample buffer and subjected to SDS–PAGE and autoradiography.

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